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Evaluation of the effect of short-term treatment with the integrase inhibitor raltegravir (Isentress^{TM}) on the course of progressive feline leukemia virus infection

Inaugural-Dissertation

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ASAT	aspartate aminotransferase
ALAT	alanine aminotransferase
AZT	3‘-azido-2‘,3‘-dideoxythymidine
b.i.d.	bis in die (twice daily)
EC_{50}	half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
FeLV	feline leukemia virus
FFU	focus forming units
FIV	feline immunodeficiency virus
HIV	human immunodeficiency virus
IC_{50}	half maximal inhibitory concentration
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
PMEA	9-(2-phosphonylmethoxyethyl) adenine
SIV	simian immunodeficiency virus
SPF	specified pathogen free
TNA	total nucleic acid
UGTA1	UDP glucuronosyltransferase gene, polypeptide A1
UGT1A6	UDP glucuronosyltransferase 1 family, polypeptide A6
XMRV	xenotropic murine leukemia-related retrovirus

1. Summary

Cats progressively infected with the gammaretrovirus feline leukemia virus (FeLV) are at high risk to die within a few months to years due to several disease symptoms that range from immune suppression, to anemia and lymphoma/ leukemia. Up to now, there is no specific drug available to treat FeLV infection.

In the present study an *in vivo* experiment was performed to investigate if a short-term treatment with the integrase inhibitor raltegravir can effectively suppress FeLV replication, allowing the immune system to overcome the infection and therefore progressively infected cats to turn to a latent status (regressive infection).

After demonstrating the high tolerance of raltegravir in three healthy specified pathogen-free cats, seven progressively infected cats were treated during nine weeks (40 mg b.i.d. for 6.5 weeks and 80 mg b.i.d. for 2.5 weeks). A significant decrease in plasma viral RNA loads was observed, indicating the ability of raltegravir to reduce viral replication. However, a complete control of viremia was not achieved and only one cat showed a marginal antibody response against whole virus, which was probably insufficient to overcome the infection. Further investigations are needed to find an optimized treatment against FeLV, i.e. an ideal dosage, compound or combination of compounds has to be identified.

2. Zusammenfassung

Für progressiv mit felinem Leukämievirus (FeLV) infizierte Katzen besteht ein hohes Risiko innerhalb von Monaten bis Jahren an den Folgen diverser Erkrankungserscheinungen wie Immunsuppression, Anämie oder Lymphomen/ Leukämie zu sterben. Bislang ist noch kein spezifisches Medikament erhältlich, um FeLV zu therapieren.

In der aktuellen Studie wurde ein *in vivo* Experiment durchgeführt, um zu eruieren, ob eine Kurzzeittherapie mit dem Integrase Inhibitor Raltegravir die FeLV-Replikation effektiv unterdrücken kann, damit das Immunsystem die Infektion überwinden und progressiv infizierte Katzen zu einem latenten Status bringen kann (regressive Infektion).

Nachdem eine sehr gute Verträglichkeit von Raltegravir bei drei gesunden spezifisch-pathogen freien (SPF) Katzen gezeigt werden konnte, wurden sieben progressiv infizierte Katzen während neun Wochen behandelt (40 mg b.i.d. für 6.5 Wochen und 80 mg b.i.d. für 2.5 Wochen). Es wurde ein signifikanter Rückgang der viralen RNA-Last im Plasma beobachtet, was für die Fähigkeit von Raltegravir spricht, die virale Replikation zu reduzieren. Eine vollständige Kontrolle der Virämie wurde allerdings nicht erreicht und nur eine Katze zeigte eine grenzwertige Antikörperantwort gegen das ganze Virus, welche vermutlich nicht ausreicht, um die Infektion zu überwinden.

Weitere Untersuchungen sind notwendig, um eine optimale Therapie gegen FeLV zu finden, d. h. eine ideale Dosis, ein ideales Präparat oder eine Kombination von Präparaten.

3. Introduction

Feline leukemia virus (FeLV) is a gammaretrovirus with worldwide distribution, affecting domestic cats and also some free-living felids, among them the Iberian lynx and the Florida puma (1-3). Of the four known outcomes of infection, progressive infection, regressive infection with or without antigenemia and abortive infection (4) , especially the persistently viremic cats (progressive infection) are at risk to die due to hematopoietic disorders, the consequences of immunodeficiency or fatal neoplasia (5).

Although the prevalence seems to have decreased in Switzerland probably due to vaccination programs and a consistent management to detect infected cats (5), there are still countries with high prevalences (6-8).

So far, there are no possibilities to treat FeLV infection with a specific medication. In one study, feline interferon omega was shown to inhibit FeLV *in vitro* and to have immunomodulatory activities, leading to an improvement of clinical scores and an extended survival time *in vivo* (9). However, there was no direct demonstration of antiviral effects *in vivo*; no viral parameters were measured throughout the study. In a recent clinical study on three FeLV-infected cats and seven cats infected with feline immunodeficiency virus, recombinant interferon omega seemed to have immunomodulatory properties, but no antiviral effect (10). *In vivo* treatment trials with the nucleoside analogue 3‘-azido-2‘,3‘-dideoxythymidine (AZT) showed an inhibitory effect, but high toxicity as well, which resulted, among others, in non-regenerative anemia (11). Recent antiretroviral agents emerging for the treatment of human immunodeficiency virus (HIV), namely the integrase strand transfer inhibitors raltegravir and elvitegravir, were tested *in vitro* against lentiviruses, alpha-, beta- and gammaretroviruses of different species (12). Raltegravir is the only integrase inhibitor so far available on the market, which exhibited good efficiency against gammaretroviruses.

It showed excellent inhibitory potential against the gammaretrovirus xenotropic murine leukemia-related retrovirus XMRV, a virus closely related to FeLV (13-15). An inhibitory effect on FeLV was also verified in three different feline cell lines with EC_{50} in the low nanomolar range, similar to what has previously been observed for HIV and XMRV (16). If effective, a treatment of viremic domestic cats with raltegravir would be of enormous advantage to alleviate the consequences of a progressive FeLV infection. However, due to financial and patient/ owner compliance considerations, the goal of the use of raltegravir in veterinary medicine has to be a temporally limited treatment, resulting in a complete recovery, even after treatment interruption. We speculate that when achieving a significant reduction of viral loads, the immune system of the cats may be able to overcome the viremia, turning the course of infection from progressive to regressive.

Indeed, complete suppression of viral replication after a transient period of viremia of more than a year has been documented in some cats (17), but these are very rare cases. A similar phenomenon was observed in some HIV patients, who were treated in an early phase of HIV infection. Probably due to the low viral reservoir in these patients, a full recovery of the infection was possible with no rebound of viremia even after treatment interruption (18).

The aim of the present study was to assess the tolerance of raltegravir in domestic cats and the effect of a short-term treatment with raltegravir on the course of FeLV infection in progressively infected specified pathogen-free (SPF) cats, by monitoring the clinical outcome, as well as the FeLV antigen, proviral, viral and specific antibody load in these cats.

4. Materials and methods

4.1. Treatment tolerance in healthy SPF domestic cats

To investigate the feasibility of the administration of raltegravir and to better assess the potential side effects, we first tested the drug in uninfected healthy SPF cats with a dosage in the range used in humans, i.e. about 5-10 mg/kg twice daily (19).

4.1.1 Raltegravir (Isentress™)

Isentress™ (raltegravir 400 mg film tabs, MSD Merck Sharp & Dohme AG, Luzern, Switzerland) was ground and re-encapsulated into smaller portions of 20 mg or 40 mg (Kantonsapotheke Zurich, Zurich, Switzerland). To facilitate the uptake by the cats, small gelatin capsules were used (14.4 x 5.1 mm, Inter Delta SA, Givisiez, Switzerland) and administered with the food twice daily.

4.1.2 Animals

Three healthy adult SPF cats (three to five years old, four to five kg of weight) were treated with 20 mg raltegravir b.i.d. during eight weeks followed by 40 mg b.i.d. for another seven weeks.

All animal experiments were performed according to Swiss laws and were officially approved by the veterinary office on the canton Zurich (TVB 160/2010). The cats were kept in groups under etiologically and hygienically ideal conditions as described (20) and the SPF status of the cats was verified prior to the experiment as described previously (21).

4.1.3 Sample collection and processing

Blood samples were collected under sedation (0.01 mg/kg midazolam, Dormicum®, Roche Pharma AG, Reinach, Switzerland) and 10 mg/kg ketamine, Narketan®, Vétoquinol AG, Belp, Switzerland) by jugular venipuncture using 5 ml syringes in weeks 0, 1, 2, 4, 6, 8, 9, 10, 11, 13 and 15. Blood was immediately transferred into Ethylenediaminetetraacetic acid (EDTA)- and heparin-anticoagulated tubes and hematological (white blood cell count and differential, red blood cell count and size, hematocrit, hemoglobin concentration, mean cell hemoglobin, mean corpuscular hemoglobin concentration, platelet count) and clinical-chemical parameters (total bilirubin, plasma glucose, fructosamin, blood urea nitrogen, creatinin, protein (Biuret), albumin, cholesterin, triglycerides, alkaline phosphatase, amylase, lipase, aspartate aminotransferase, alanine aminotransferase, sodium, potassium, chloride, calcium, phosphate) were assessed, using standard methods of the clinical laboratory.

The raltegravir plasma concentration of each time point was measured by high performance liquid chromatography- mass spectrometry at the University Hospital Zurich, Institute for Clinical Chemistry under conditions described (22). Heparin-anticoagulated plasma from untreated cats was used as negative control. Plasma concentrations were also determined, after raltegravir administration has been omitted for 24h and 36h, to compare elimination rates of cats with those of humans (23).

4.2 Treatment of SPF cats experimentally infected with FeLV

4.2.1 Animals

Eighteen, 14-16 weeks-old SPF male kittens (Liberty Research, Inc., Waverly, NY, USA) were kept under barrier conditions in two groups and the SPF status was verified

by testing blood, saliva and feces by PCR, RT-PCR and serology for absence of FeLV, FIV, feline parvo-, herpes-, corona-, calicivirus and feline hemotropic mycoplasmas as described (20).

4.2.2 FeLV-A virus challenge

Four aliquots of FeLV-A/ Glasgow 1 strain (24) stored at -80°C (kindly provided by Veterinary Diagnostic Services, School of Veterinary Medicine, College of Medical, Veterinary and Life Science, Glasgow University, Glasgow, UK) were thawed at 37°C, pooled (total: 17 ml, 1.1 x 10^6 FFU/ml) and the virus diluted on ice to 23 ml using RPMI cell culture medium (Invitrogen AG, Basel, Switzerland). Subsequently, 1 ml was aspired in a syringe and stored on ice until use.

At the age of 19-21 weeks, each cat was infected intraperitoneally with 800‘000 FFU (in 1 ml) of this virus.

In week 6 post-infection a second virus challenge with 1.7 x 10^6 FFU (in 1.8 ml) was performed intraperitoneally in ten cats with only transient viremia. Origin and virus preparation was identical to the first challenge.

4.2.3 Treatment

Fifteen weeks after the first virus challenge, seven progressively infected cats were treated with 40 mg b.i.d. (corresponding to 10-15 mg/kg b.i.d.) raltegravir during six and a half weeks. Because none of the cats had cleared antigenemia by then, the dose was increased to 80 mg b.i.d. (20-25 mg/kg b.i.d.) for another two and a half weeks, starting in week 7 of the treatment.

Historical data of previous experiments was used to compare the course of infection of untreated progressively infected cats with the seven treated cats of the present study.

For this experiment, Isentress^{TM} was ground and re-encapsulated and the following gelatin capsules were used: 19.0 x 6.6 mm, containing 40 mg raltegravir, filled up with 320 mg of lactose and 14.4 x 5.1 mm as described above (4.1.1).

4.2.4 Sample collection and processing

Blood samples were collected under sedation in weeks 0, 1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15 post-infection (p.i.), in weeks 1, 2, 3, 4, 5, 6, 7, 8, 9 during the treatment and in weeks 1, 2, 4, 6, 8 post-treatment. Hematological and clinical-chemical parameters were assessed as described above (4.1.3).

The raltegravir plasma concentration was measured in weeks 0, 3, 6, 9 of the treatment and in week 1 post-treatment (4.1.3).

4.2.5 Detection of FeLV viral DNA in blood and viral RNA in plasma

For determination of FeLV blood viral DNA loads, total nucleic acids (TNA) were extracted from a blood volume containing 10^6 white blood cells, or from a maximum volume of 100 µl, using the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche diagnostics AG, Rotkreuz, Switzerland) according to manufacturer's instructions. All volumes were adjusted to 100 µl with phosphate-buffered saline (PBS). Negative controls consisting of 200 µl of PBS were concurrently prepared with each batch of samples to monitor for cross-contamination. The extracted TNA were analysed by real-time PCR for FeLV DNA loads as described (25) on a ABI PRISM 7700/7500 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland). PCR runs were performed with negative controls consisting of 5 µl of nuclease-free water.

TNA were extracted from 200 µl of plasma using the MagNa Pure LC Total Nucleic Isolation Kit according to manufacturer's instructions and quantified by real-time RT-PCR for FeLV plasma viral RNA loads as described (25).

4.2.6 Detection of FeLV virus antigen p27 by ELISA

FeLV viremia was determined by p27 double antibody sandwich ELISA as described (26). All samples were tested in duplicates. Results were calculated as the percentage of a positive control (culture supernatant of FL-74 feline lymphoblastoid cell line permanently infected with FeLV), which was assayed with every plate. Values above 4% were considered to be positive. In agreement with the European Pharmacopoeia (2005), a cat was designated as progressively infected when FeLV p27 antigen was positive for three consecutive weeks or on five occasions between weeks 3 and 15 p.i.

4.2.7 Antibody Assays: FeLV whole virus and p45 antibody ELISAs

The plasma samples were analysed for the presence of anti FeLV whole virus and anti FeLV p45 antibodies by ELISA, using 100 ng of p45/ well and 100 ng of gradient purified FL-74 FeLV with a dilution of 1:100 (whole virus ELISA) and 1:200 (p45 ELISA) as described (27, 28). In each assay pooled SPF sera (= negative control) and pooled sera collected from FeLV immune cats (= 100% positive control) were tested. Values above 25% of the positive control were considered to be positive.

4.2.8 Statistics

Statistical analyses were carried out using R software version 2.14.0 (the R Foundation for Statistical Computing, Vienna, Austria). Longitudinal effects (time) of the treatment

on viral RNA, viral DNA and p27 loads were compared by multivariate analysis of variance (MANOVA). A p-value < 0.05 was considered to be statistically significant.

For the correlation analysis, the Spearman rank correlation test was used (p_S), performed with Graph-Pad Prism (GraphPad Software, Version 3.0. San Diego, CA, USA). A p-value < 0.05 was again considered to be statistically significant.

5. Results

5.1. Raltegravir-associated side effects in healthy SPF cats

Cats showed neither changes in their behavior nor any clinical symptoms e.g. apathy, vomiting or diarrhea during the 15 weeks of treatment. The hematological and clinical-chemical parameters remained unchanged and within the reference range.

Raltegravir plasma concentrations ranged from 46 to 1040 nM at 20 mg b.i.d., and from 151 to 1057 nM at 40 mg b.i.d. Plasma levels of raltegravir were almost undetectable after twenty-four hours (week 10) and thirty-six hours (week 11) of drug withdrawal.

Both, 20 mg and 40 mg dosages, resulted in sufficient plasma concentrations to guarantee a good efficacy (at least 99% inhibition *in vitro*) (16). Relatively high differences in the raltegravir plasma concentrations between the cats and also a high variability between the respective time points were observed in all cats (Fig. 1).

Because of the excellent tolerance and higher plasma concentration reached, we opted for the 40 mg regimen for the test in experimentally infected domestic cats.

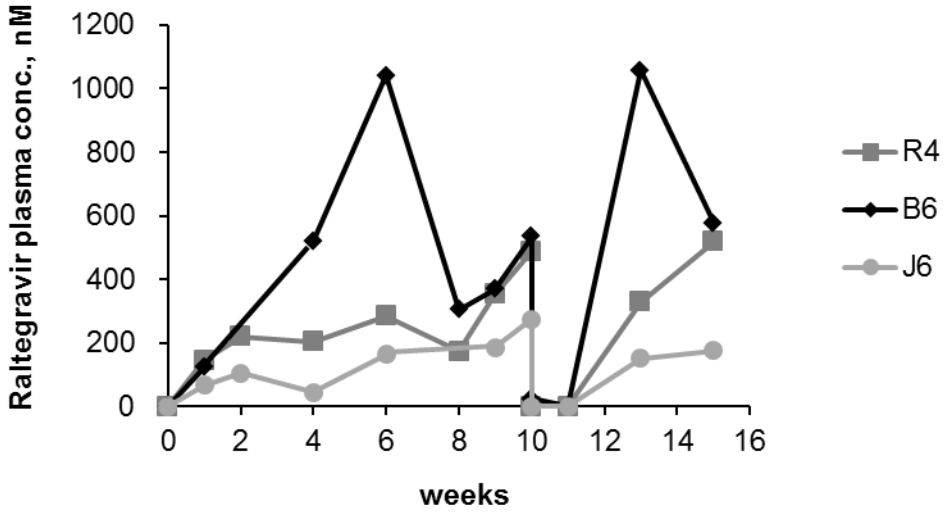


Fig. 1 Raltegravir plasma concentration (nM) of three adult SPF cats measured during a treatment period of 15 weeks.

Weeks 1-8: 20 mg raltegravir b.i.d. Weeks 9-15: 40 mg raltegravir b.i.d.

Data points in weeks 10 and 11 represent plasma values after 24h and 36h of raltegravir withdrawal.

Missing data in week 2 (cat B6) and in week 8 (cat J6) are due to the collection of high blood volumina in the beginning of the experiment and therefore a treatment-unrelated excessive decrease of the hematocrit (cat B6) and to anesthesia failure (cat J6).

5.2. Treatment of SPF cats experimentally infected with FeLV

5.2.1 FeLV infection outcome

According to the results obtained by p27 ELISA, seven out of eighteen cats developed a persistent viremia (Fig. 2). Ten cats had a transient outcome of infection. All infected cats became FeLV provirus and viral RNA positive, indicating that FeLV infection had occurred in all of them (data not shown); one cat died four weeks p.i. with FeLV-related symptoms. Furthermore, there was no change in the infection-status after a second challenge of the ten cats, which induced only transient viremia.

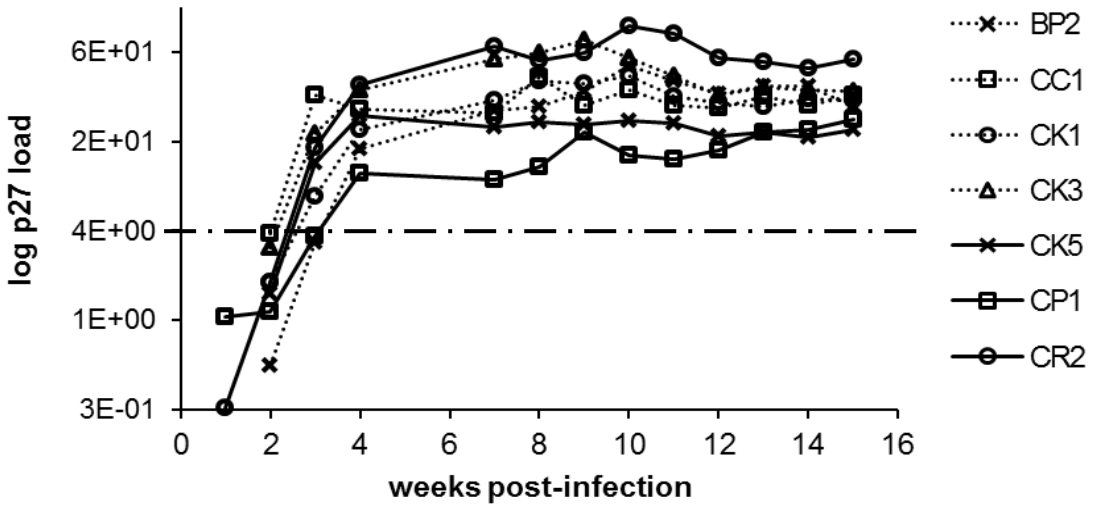


Fig.2 Plasma FeLV p27 antigen loads of the seven viremic cats until week 15 p.i. The horizontal dotted/broken line represents the threshold for p27 positive results (≥ 4% of the positive control).

5.2.2 Treatment outcome

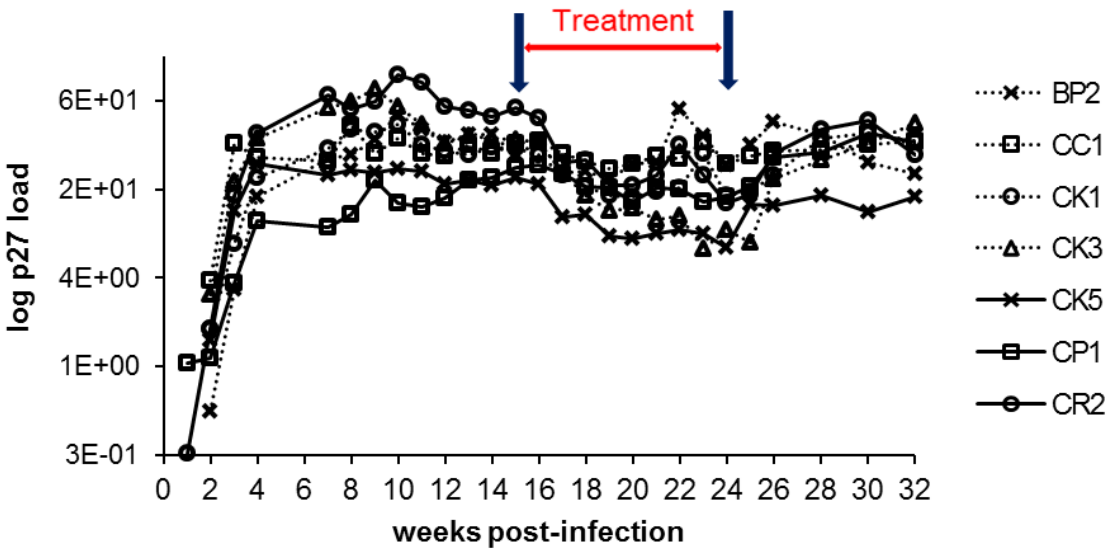
Raltegravir treatment of the seven progressively infected cats was started in week 15 p.i. Because after six weeks of treatment (week 22 p.i.) no cat had turned completely negative for p27 antigen, a twofold higher dosage (80 mg b.i.d., corresponding to 20-25 mg/kg) was administered to all seven cats for another two and a half weeks, starting in week 7. Although, such a high dosage has never been tested before, it was also well tolerated by the cats and no side effects were observed. FeLV p27 antigen loads, anti-FeLV whole virus and p45 antibodies, as well as FeLV provirus and plasma viral RNA loads were determined to assess the inhibitory potential of the treatment (Fig. 3).

In all seven cats, a significant correlation between the FeLV p27 antigen loads and the plasma viral RNA loads was observed ($p<0.0001$), (Fig. 4).

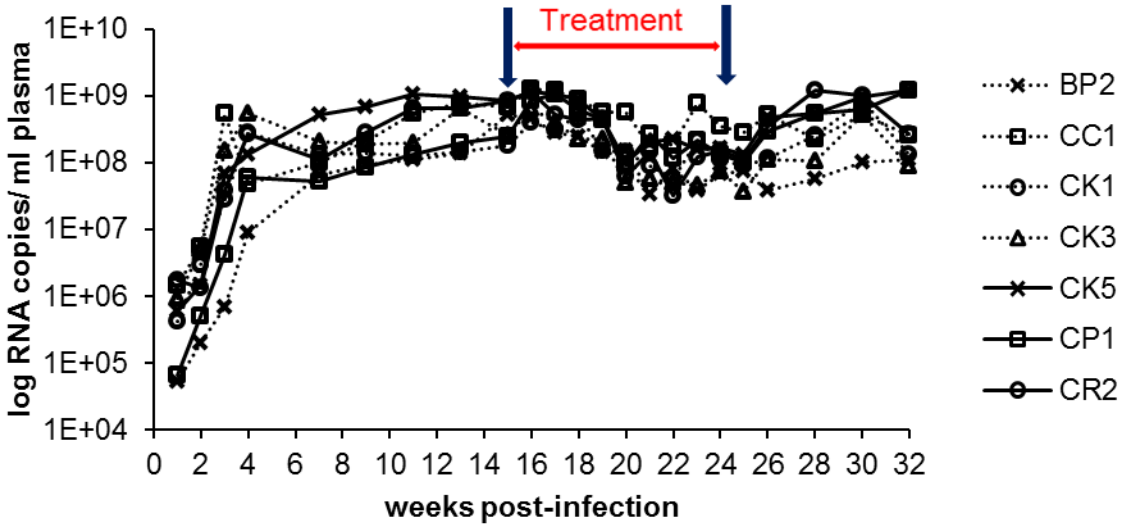
Comparison with data from previous experiments using similar challenge doses (500'000 FFU) of the same virus in experimentally infected SPF cats (29) showed that treatment with raltegravir was effective in reducing viral RNA loads: $p_{MANOVA}= 0.048$ for weeks 11, 15 (baseline), 17, 21 and 24 (treatment) p.i., $p_{MANOVA}= 0.031$ for weeks 11, 15 (baseline), 17 and 21 (treatment) p.i. The viral load reduction was approximately fivefold and became first apparent in week 5 of the treatment (Fig. 3b, Fig. 4, Table 1).

Despite the high correlation between RNA and p27, the effect was only marginal for p27 antigen: $p_{MANOVA}= 0.087$ for weeks 11, 15 (baseline), 17, 21 and 24 (treatment) p.i., with two out of seven cats turning to nearly negative values (Fig. 3a). No effect by raltegravir was observed on provirus loads: $p_{MANOVA}= 0.668$ for weeks 11, 15 (baseline), 17, 21 and 24 (treatment) p.i. (Fig. 3c).

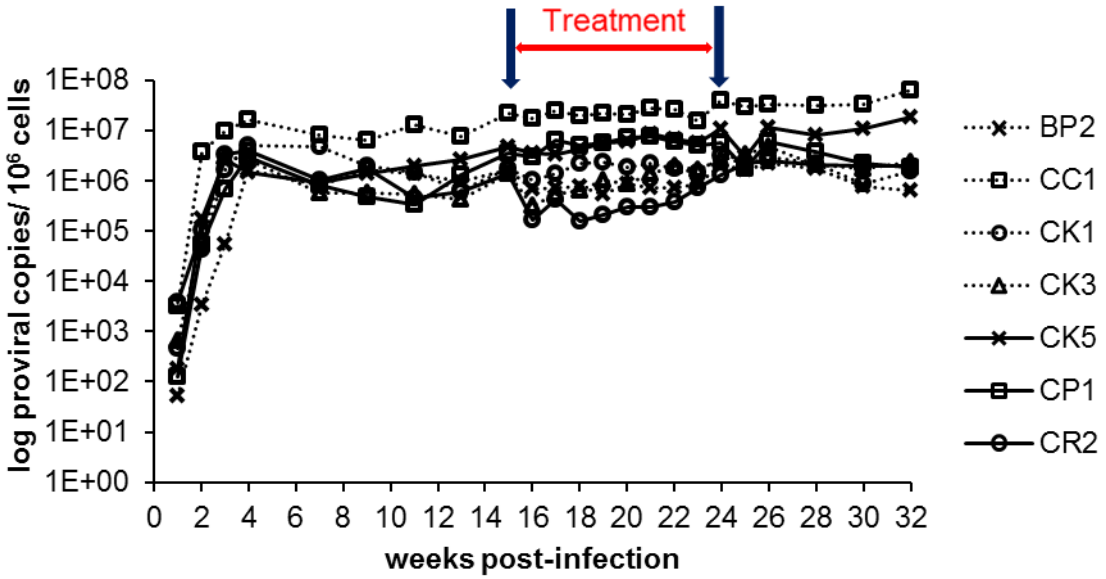
The anti FeLV p45 antibody levels remained below the threshold for positive results at any time point (data not shown). Only cat BP2 turned positive for anti FeLV whole virus antibodies in response to treatment (week 2 to 8 post-treatment, Fig. 3d). Cats CK5 and CR2 showed high anti FeLV whole virus antibody titers in week 4 p.i., but one week later they turned back to negative values again, with no change until the end of the experiment (Fig 3d).



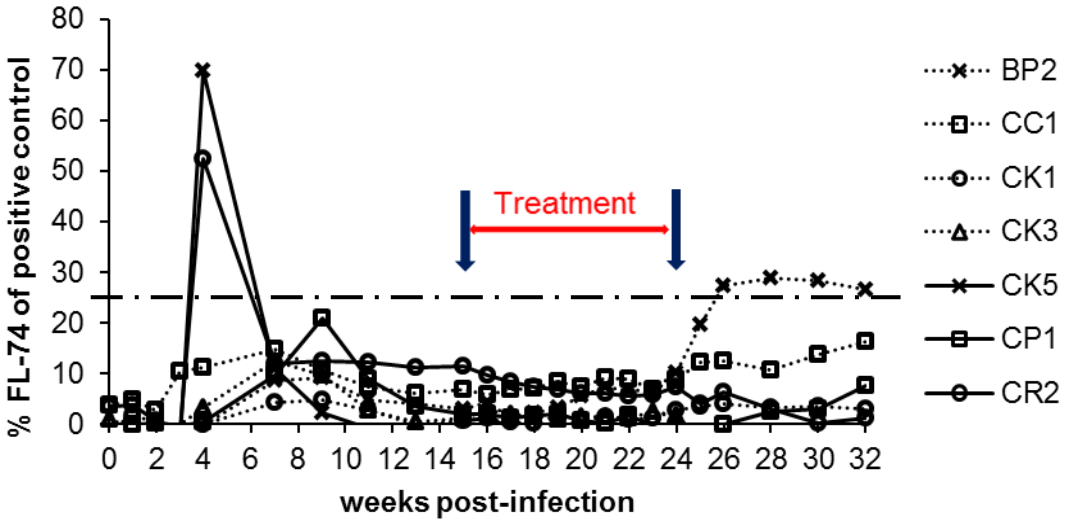
(a)


Treatment
log RNA copies/ml plasma
1E+10
1E+09
1E+08
1E+07
1E+06
1E+05
1E+04
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
weeks post-infection
BP2
CC1
CK1
CK3
CK5
CP1
CR2

(b)

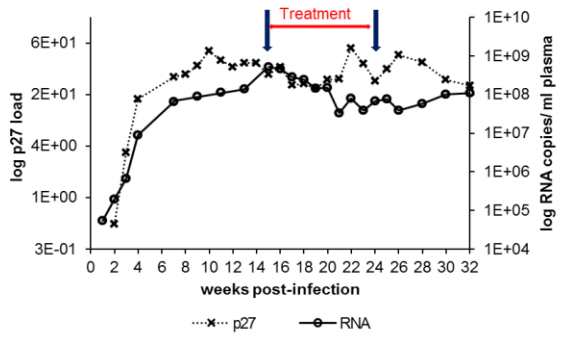

Treatment
log proviral copies/ 10^6 cells
1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
1E+02
1E+01
1E+00
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
weeks post-infection
BP2
CC1
CK1
CK3
CK5
CP1
CR2

(c)

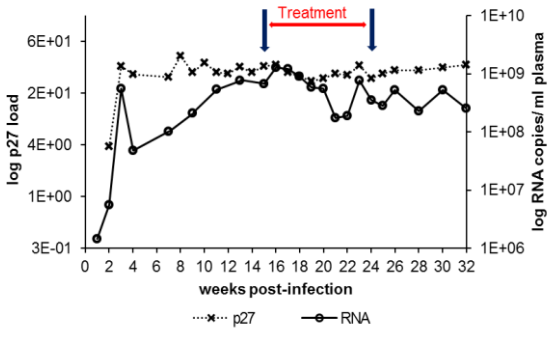


(d)

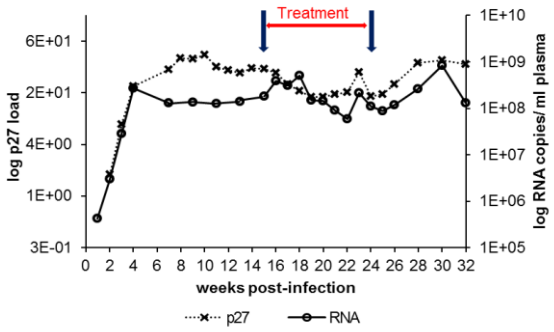
Fig.3 Plasma FeLV p27 antigen loads (a), viral RNA loads in plasma (b) and proviral DNA loads in blood (c) of the seven treated cats during the whole experiment. (d) Anti FeLV whole virus antibodies of the seven treated cats. The horizontal dotted/broken line represents the threshold for positive results (≥ 25% of the positive control).



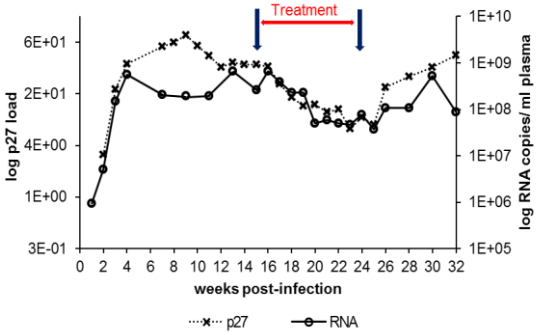
(a)



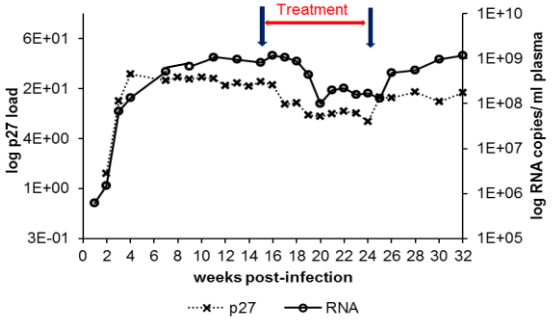
(b)



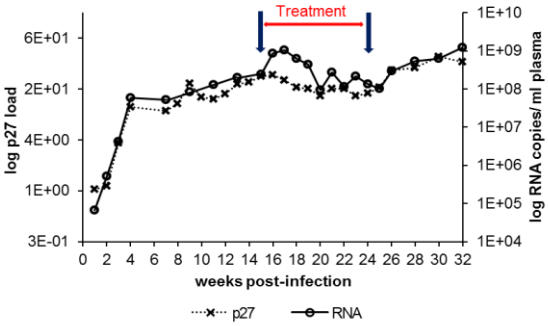
(c)



(d)



(e)



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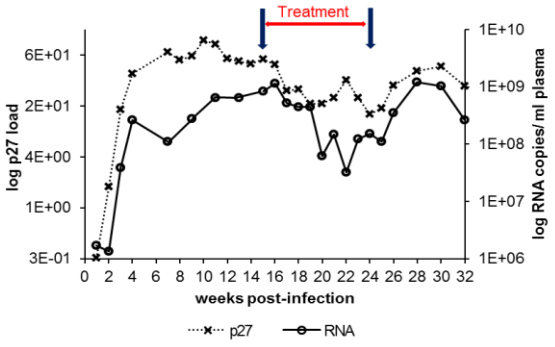

(g)

Fig. 4 Course of viral RNA and p27 loads for all cats plotted seperately (a-g: cat BP2, CC1, CK1, CK3, CK5, CP1, CR2, respectively). Treatment start was in week 15 p.i., interruption took place in week 24 p.i.

Table 1: Viral RNA load reduction compared to baseline (fold) of the seven progressively infected cats, during and after treatment. Gray cases highlight values > 3.0. WPI: weeks post-infection. BL: baseline

	BL	TREATMENT week									INTERRUPTION week				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**1**	**2**	**4**	**6**	**8**
WPI	15	16	17	18	19	20	21	22	23	24	25	26	28	30	32
BP2	1.0	1.1	1.8	2.1	3.5	3.4	15.2	6.3	13.3	7.6	6.8	13.1	8.8	5.1	4.7
CC1	1.0	0.5	0.5	0.7	1.2	1.2	3.8	3.5	0.9	1.9	2.4	1.3	2.9	1.3	2.6
CK1	1.0	0.5	0.6	0.4	1.2	1.2	2.0	3.1	0.8	1.6	2.1	1.5	0.7	0.2	1.4
CK3	1.0	0.4	0.7	1.1	1.1	5.2	4.4	5.1	5.6	3.4	6.9	2.4	2.4	0.5	3.0
CK5	1.0	0.7	0.8	0.9	1.9	8.3	4.1	3.7	5.2	4.9	6.4	1.7	1.5	0.9	0.7
CP1	1.0	0.3	0.2	0.4	0.6	2.6	0.9	2.1	1.1	1.9	2.4	0.8	0.5	0.4	0.2
CR2	1.0	0.7	1.6	1.9	1.9	13.4	5.7	26.2	6.8	5.6	7.6	2.4	0.7	0.8	3.1
Mean	1.0	0.6	0.9	1.1	1.6	5.1	5.2	7.2	4.8	3.8	4.9	3.3	2.5	1.3	2.2
St Dev	0.0	0.3	0.6	0.7	0.9	4.5	4.7	8.5	4.5	2.3	2.5	4.3	2.9	1.7	1.6

5.2.2.1 Raltegravir plasma concentration

Raltegravir plasma concentration ranged from 122 to 1413 nM at 40 mg b.i.d. and from 655 to 1704 nM at 80 mg b.i.d. (Fig. 5). Cats CC1, CK1 and CK3 exhibited a high variability between the respective time points.

One week after treatment interruption raltegravir was barely detectable (Fig. 5).

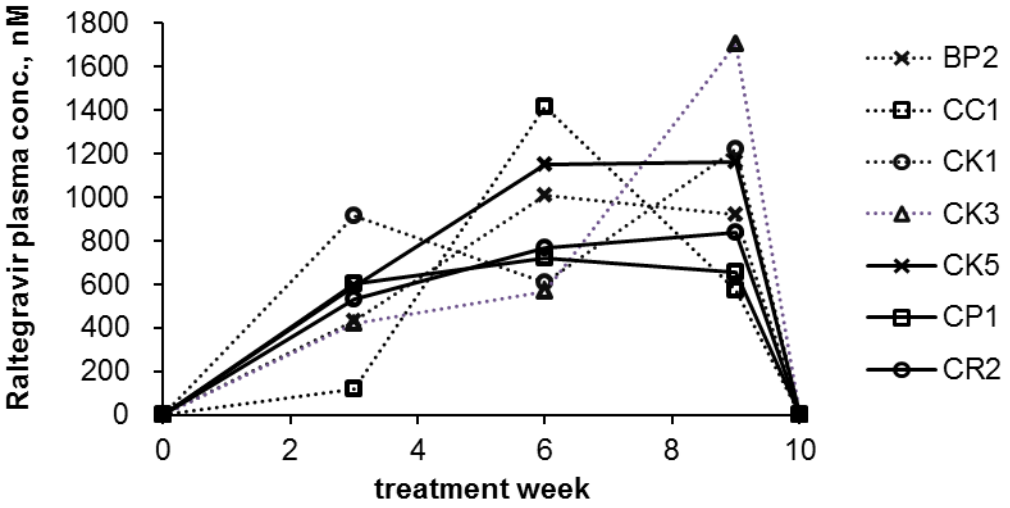

Fig. 5 Raltegravir plasma concentration (nM) of the seven progressively infected cats, measured in weeks 0, 3, 6 and 9 of the treatment and one week post-treatment (week 10). Time points 3 and 6 show the concentration where 40 mg raltegravir was administered twice daily, while week 9 corresponds to a twofold higher dosage (80 mg b.i.d.).

5.2.3 Post-treatment outcome

Raltegravir treatment was interrupted at the end of week 24 p.i. and the course of infection was monitored for another eight weeks. Plasma viral RNA loads started to increase again, returning to the same levels found prior to treatment within four weeks (Fig. 3b, Table 1), except for cat BP2, which exhibited the best overall response and was the only cat found positive for anti FeLV whole virus antibodies (Fig. 3d, Table 1). In this cat, RNA load reduction was still approximately fivefold at the end of the study, and p27 seemed to be decreasing as well.

6. Discussion

The aim of the present study was to investigate whether the integrase inhibitor raltegravir has an inhibitory effect on FeLV *in vivo*, and if the magnitude of the inhibition enables cats to mount an adequate immune response, thus overcoming the consequences of the infection. For this purpose we first tested the drug in three healthy SPF cats, to ensure that raltegravir treatment does not cause severe side effects. The main metabolic pathway of raltegravir is by glucuronidation, well-known to be poor in domestic cats (30, 31). Hence, it was expected that tolerance to this drug may be worse than in humans or other species. However, the defective enzyme of glucuronidation in the cat (related to the absence of expression of UGT1A6) (30) is dissimilar to the enzyme mainly responsible for the glucuronidation of raltegravir (UGTA1) (23). Therefore, this metabolic defect in cats may not play a very important role for the elimination of raltegravir. Observations in other species confirmed an overall high tolerance to raltegravir. Dogs receiving raltegravir orally for one year at a dosage up to nine-fold above the 400 mg b.i.d. clinical dose (corresponding to ~360 mg/kg b.i.d.) used in humans did not show liver toxicity (19). Analogue results were seen in rats after six months of oral administration at up to 4.8-fold higher dosage than the 400 mg b.i.d. clinical dose (~2400 mg/kg) (19). In humans, twice the dosage (~12 mg/kg) is well tolerated (19) . These observations are consistent with our findings in SPF cats, where absolutely no side effects were observed during the whole period of 15 weeks, although we also administered a twofold higher dosage of 40 mg twice daily. Moreover, a fourfold higher dosage used for two and a half weeks in the major project was very well tolerated, too. In addition, a recent *in vitro* study showed, that raltegravir failed to induce toxicity even at concentrations 280-fold higher than the IC_{50} for antiviral activity in

Crandell Reese feline kidney cells (CrFK) (32), thus confirming the results obtained previously in our laboratory (16).

To determine the effect of raltegravir in progressively infected cats, 18 kittens were infected intraperitoneally with 800'000 FFU FeLV-A Glasgow 1 strain. According to previous observations, 14 to 17 cats were expected to show a progressive infection (3, 33-35). Unfortunately, only seven cats developed a progressive infection in the current study. An explanation for this unanticipated development may be a sudden drop in quality of the challenge virus. However, the virus, shipped in dry ice directly from the University of Glasgow a few days before experimental infection, was harvested, stored and the quality assessed under the same conditions as in previous experiments. Therefore, an extremely poor quality of the virus as a cause of the failure of obtaining enough progressively infected cats is rather unlikely, especially because already 50'000 FFU are sufficient to cause a progressive infection in 8 of 10 cats, infected at the age of 17-19 weeks (29). In addition, one cat died because of FeLV within a few weeks after infection, confirming that the virus stock used had a good infectivity. Alternatively, high endogenous FeLV loads in the cats may have been responsible for the poor progressive infection rate. Endogenous retroviruses can lead to partial resistance by receptor interference (36-38) and a similar effect has been hypothesized for FeLV as well (39), although not yet statistically validated.

To achieve a higher number of progressively infected cats, a re-infection of the ten transiently viremic cats with 1.7×10^6 FFU FeLV-A Glasgow 1 strain was performed six weeks after the first challenge. Surprisingly, there was no change in the infection status, indicating an already developed protective immune response in these cats (40).

Based on the low number of treatable cats, there was no possibility to include an equal control group in this study. Therefore, we decided to treat all the progressively infected cats and to compare the outcome with historical data (29).

During the nine weeks of treatment a decrease in plasma viral RNA loads was observed, indicating the ability of raltegravir to reduce viral replication. The decrease of plasma viral RNA loads was in the range of one log10 for four of the seven cats (Table 1). In humans, raltegravir therapy achieved, in general, a higher decrease (at least 2 log10), but this was always as a combination therapy (19), and the effect of raltegravir alone was difficult to discern. In three SIV-infected macaques, the effect was of around 3 log10 reduction after four weeks treatment to undetectable levels (41). Thus, raltegravir seems to be less efficient in reducing replication in FeLV than in HIV or SIV-infected individuals. RNA levels in FeLV-infected cats are in the range of 10^8-10^9 copies/ml, while in the case of HIV and SIV the range is much lower: 10^3-10^5 copies/ml. Thus, viral reservoirs seem to be much more active for FeLV than for HIV/SIV, and this may explain the lower magnitude of the reduction in RNA loads. The non-significant change in p27 loads may be the consequence of the lower sensitivity of the test: only changes in p27 correlated with a much higher decrease in RNA loads may be statistically significant.

As observed e.g. for SIV in macaques (41), viral DNA levels remained unchanged during treatment. This does not necessarily mean that the viral reservoir was unaffected by the treatment. The fivefold decrease in RNA loads shows that new cells are steadily infected in progressively infected cats even after RNA loads have reached a steady state. Integrase inhibitors do not prevent DNA synthesis, but only its integration into the host's genome. Virus, infecting cells without integrating DNA, is unable to generate new RNA. Unchanged DNA levels may be due to the fact, that the qPCR test for FeLV is not

able to differentiate integrated (provirus) from unintegrated viral DNA. Nevertheless, once occurred, the integration process is irreversible. Therefore, treatment in the early phase of infection is really important, as exemplified in humans, where viral DNA levels were lower after treatment with raltegravir in HIV primary vs. chronic infection (42).

Only in one cat (BP2) a marginal anti FeLV whole virus antibody response could be observed shortly after treatment interruption, but no anti p45 antibody response was detected in any cat at any time point. According to our knowledge, anti p45 antibodies are the most important to generate an effective immune response against FeLV (40). Anti FeLV whole virus antibodies can also protect from viremia, when titers are high enough and present for a longer period of time (> 3 months) (3). In this case, antibody concentrations of 27-28% of the positive control are a too low titer to change the course of infection and turn to a latent status in the time frame we monitored. Nevertheless, if persistent over a long time, the anti FeLV whole virus antibody response may lead to regressive infection, especially if combined with a continuation of the therapy, that would keep the viral replication at lower levels: cat BP2 was, indeed, the one that best responded to therapy in terms of RNA load reduction (Table 1), which was still approximately fivefold at 8 weeks after interruption of treatment. Therefore, long-term monitoring of this cat and, eventually, a continuation of the therapy will be crucial in determining if raltegravir treatment can, under some circumstances (e.g. cats with similar genetic background and/ or gene expression patterns), really induce a regressive infection.

By week 4 after treatment interruption, plasma viral RNA loads had increased again in all seven cats, returning to the levels assessed before treatment in six of them. Although the short-term treatment was not sufficient to completely stop viremia, the course

observed after treatment interruption is clear evidence for an inhibitory effect of raltegravir.

Further investigations are needed to better assess the effect of raltegravir. Although - based on the values of raltegravir plasma concentration - the use of a higher dosage is considered possible, there is no guarantee that a higher concentration in plasma and therefore a stronger effect of the treatment can be achieved. It seems that saturation occurred in some cats after administering the fourfold higher dosage of 80 mg b.i.d., leading to stable plasma concentrations. A variable genetic background and metabolism of each cat are presumably responsible for the inhomogeneous outcome.

The development of novel integrase inhibitors opens the door to further interventions. Elvitegravir (43) and so called “new generation”- compounds like dolutegravir (S/GSK 1349572) (44) or MK-2048 could be taken into account for further analyzes. MK-2048 was examined in healthy humans, but is not yet available on the market (45). Dolutegravir is actually in phase III clinical trials (46). Lower dosages and probably a single-day administration are effective using this compound (47-49). In addition, a more intimate binding mode with the viral integrase compared to raltegravir and an improved resistance profile are beneficial (50). First, an *in vitro* examination in feline cells would be necessary to approve potentially similar qualities for the application in cats.

Other classes of HIV drugs like protease inhibitors were shown to be ineffective against XMRV (14) and therefore rather ineligible for the use as an anti-FeLV drug. Reverse transcriptase inhibitors are problematic for the use in cats, because they are often only efficient near the cytotoxic threshold, as it also has been shown *in vivo* in cats treated with AZT or 9-(2-phosphonylmethoxyethyl) adenine (PMEA) (9).

Due to the fact, that a short-term treatment was not sufficient to completely stop viremia, a longer-term treatment like in HIV patients may be necessary also for cats to prevent death by progressive FeLV infection. Such a treatment is difficult to carry out, because the administration of capsules to cats is not always easy and the medicament plus the re-encapsulation are expensive and not affordable for everyone, since there is no specific veterinary market for these compounds.

Nevertheless, the current study displays a first step into the right direction to achieve an effective treatment against FeLV with recent antiretroviral compounds. It is conceivable that future studies will explore the combination of several of them rather than single therapies. Indeed, a combination with peptide FeLV envelope inhibitors (51), immune system boosters like interferon omega (10) and CpG oligonucleotides (52, 53), drugs that can deplete early T-cell infection reservoirs (54, 55), and more efficient integrase inhibitors may be more effective in reducing viral replication than the individual compounds alone. Still, for the reasons mentioned above, the endpoint of a test of these compounds *in vivo* is not only the drastic reduction of viral loads, but the induction of a state of regressive infection in the cats through a short-term treatment of a few months.

7. Conclusion

Although a short-term treatment with raltegravir is not sufficient to completely stop viremia in cats persistently infected with FeLV, a significant decrease in RNA loads indicates an influence on viral replication. An optimized treatment against FeLV, composed of the ideal dosage, compound or combination of compounds has to be identified in further investigations.

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